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An. gambiae and An. arabiensis, the primary African vectors of human malaria, are reproductively isolated but morphologically indistinguishable species. They are sympatric and probably not equally involved in all cases of malaria transmission. They are only reliably distinguished cytogenetically, thus making epidemiological studies difficult. Under a previous contract we developed an RFLP assay based upon a ribosomal DNA probe which distinguished all member species in the complex. The present studies utilized this RFLP to design species-specific oligonucleotides which are used to probe dot blots. Thus the need for DNA extraction, restriction enzyme digestion, and the running and blotting of gels are eliminated. A species-specific restriction fragment from An. gambiae was also studied as a means for augmenting the oligonucleotide probes. The oligonucleotide probes were shown to be species-specific and useful for a rapid dot blot assay.

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Victoria Finnerty 8.9.89
PI Signature Date

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1. Statement of Problem under Study

Malaria is the most debilitating disease in Africa today: There are approximately 1 million deaths annually, nearly all of which are infants or young children (1). Malaria is most acute in subSaharan Africa where, in addition to the mortality, the disease presents an enormous obstacle to social and economic development. Malaria incidence continues to increase due to many factors which include drug resistance in the parasite and insecticide resistance in the mosquito vector (2). Cloroquine resistant parasites have, in particular, led to many cases of malaria in immunologically naive visitors to areas where malaria is endemic. Changes in agricultural practices, such as new irrigation systems, as well as climatic changes have also led to increases in the incidence of malaria infections (3,4). Two of the principal vectors of human malaria in Africa are An. gambiae and An. arabiensis. These species are members of a reproductively isolated complex of species that consists of An. gambiae, An. arabiensis, An. melas, An. merus, An. quadriannulatus, and An. bwambe. Members of the species complex are found throughout Africa, but An. melas and An. merus use only salt water breeding sites (5). Despite wide differences in habitat and separation by vast geographical distances, the species have no morphological characters that allow reliable designation as to species (6).

The species differ in behavior and preferred habitat. Moreover, there is evidence suggesting that the two major vector species may not be equally involved in malaria transmission, depending upon the season and location (5). Therefore, one of the requirements for epidemiological studies of these insect vectors is to determine whether an individual female mosquito is infected with the malaria parasite, and also, to what species does she belong. The latter consideration is most pressing for studies of habitat and reproductive behavior which provide information essential for the design of various control strategies. Thus far, the only reliable means of distinguishing among the members of this complex were differences in polytene chromosome banding patterns as observed in either larval salivary gland or adult female ovarian nurse cell tissues (7,8). For field-caught specimens a female would have to be blood fed at least once by the experimenter to insure that ovarian nurse cells could produce the degree of polyteny required for examination. This method is cumbersome and requires a degree of technical expertise. It cannot readily be used for large numbers of specimens and cannot be applied to all life stages.

A clear requirement for epidemiological and ecological studies is therefore a rapid, sensitive, and easy means of classifying large numbers of single specimens. It is also especially important that the method be compatible with gathering other necessary information concerning individual mosquitoes, namely the presence of Plasmodium and the source of the bloodmeal. It would also be useful if the assay could be carried out on dried or alcohol-preserved specimens.

2. Background

The proposed studies are based upon using a DNA-based assay. The advantage of DNA is its stability, even when dried or in alcohol preserved tissue. In prior studies (funded by a previous contract, DAMD 17-85-5184) a strategy based upon restriction fragment length polymorphisms (RFLP) in ribosomal DNA

was adopted. The ribosomal genes were selected as a potential source of useful species diagnostic probes because they are present in approximately 500 copies/nucleus (9) and therefore would provide a much enhanced signal. This proved to be important because only a portion of the mosquito need be used for species diagnosis while the remainder can be used for ELISA assay for the Plasmodium antigen (10) and bloodmeal analysis. In addition, studies with other organisms had shown that despite the highly conserved nature of the ribosomal coding regions, other areas such as intergenic spacers were highly variable and could provide useful RFLPs (9). It is important to note here that the ribosomal genes have another distinct advantage compared to using (undefined) highly repeated sequences (possibly satellite sequences) which tend to show rapid divergence even between closely related species (11,12,13). Although this approach rapidly identifies useful divergent sequences and has already been used for the An. gambiae complex (14,15), it has a serious disadvantage because the copy number of such sequences often varies dramatically between the sexes or even among geographically different isolates of a single species (16). In short, the signal to noise ratio can become such as to give falsely negative results. As expected, this may also lead to the necessity of selecting probes to be used only for certain geographical locations or only for one sex. In order to avoid such problems the strategy chosen here was based upon a permanent, well studied feature of the genome whose copy number has never been found to vary by more than a factor of two among closely related species (17). Therefore, the initial phases of the work involved more time and effort, but have the advantage of identifying DNA sequences whose utility is certain for all members of a given species.

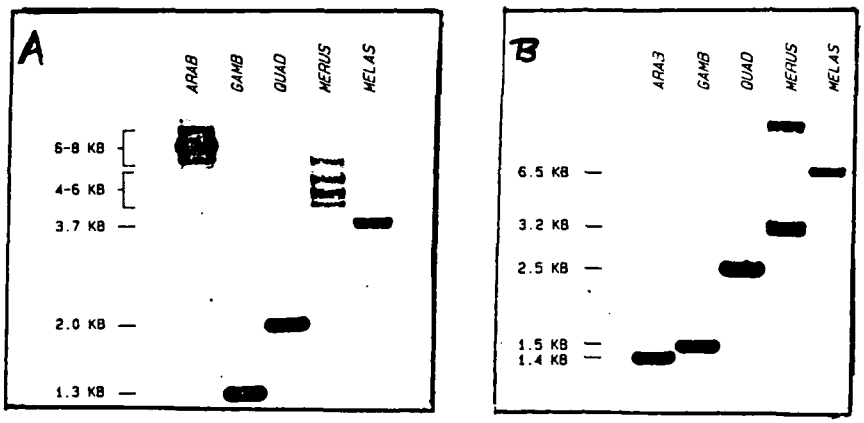
Thus we selected and studied a group of rDNA clones from an An. gambiae genomic library. The intergenic spacers were identified as a potentially useful source of sequences that would reveal RFLPs among the several species in the An. gambiae complex (18). One fragment was chosen for further study. The fragment was cloned from and initially tested on colonized mosquitoes. However, colonized specimens are subject to founder effects and thus may not be representative of the variability in natural populations. In order to be certain that we had focused on an absolutely consistent feature of each species, the reliability of the RFLP was tested using the cytogenetic method. In collaboration with Dr. Vincenzo Petrarca (Univ. of Rome), field caught specimens from Kenya and Zimbabwe were split and analyzed by both cytogenetic and DNA probe (12A) methods (19). About 97% (250) of the cytogenetically identified specimens were also identifiable by the DNA probe and in every case the species identifications were concordant. In another study, field caught specimens were tested by an enzymic typing method (20) and the DNA probe, and again the results were perfectly concordant. Specimens which were not scoreable by the isozyme method were classified with the DNA probes.

3. Rationale

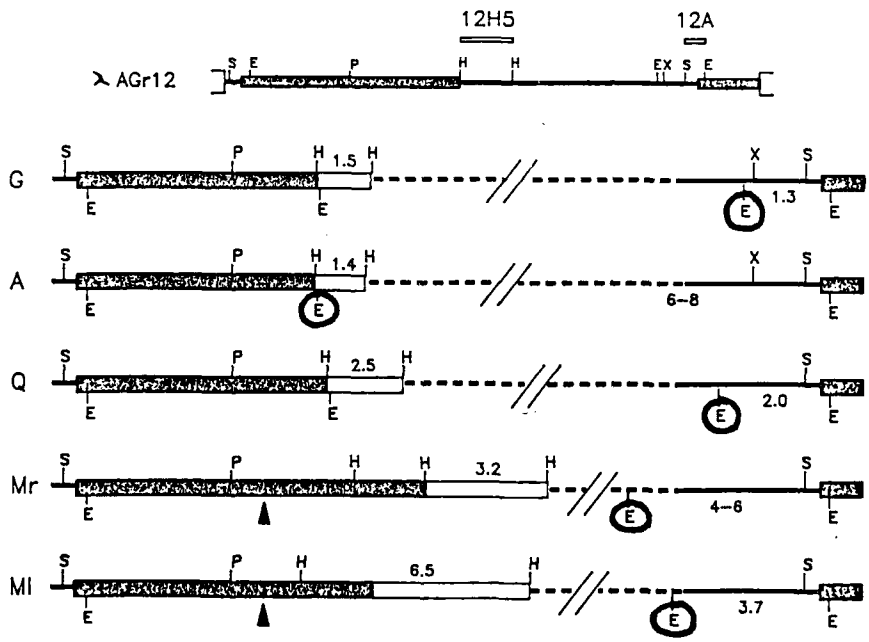
The primary goal of the present experiments was to develop a more simple and rapid method for species diagnosis. The form of the method would be a dot blot (or slot blot) which eliminates the need for DNA extraction, restriction enzyme digestion and the running of gels. The blotting step would also be eliminated.

The approach was to make use of the RFLPs we had previously studied in order to design a set of oligonucleotide probes which would positively identify only

a single species within the An. gambiae complex. In particular, since An. gambiae and An. arabiensis are the major vectors of human malaria our studies were directed at these species. The figure below illustrates two of the most useful RFLPs. The probe (12H5) used on the right hand side, blot (B) (digested with HindIII) had not been tested as extensively on field specimens, but the probe (12A), used on the left (A) (digested with EcoRI) reveals an absolutely reliable RFLP (18,19,20). Approximately one-half mosquito is used per lane.



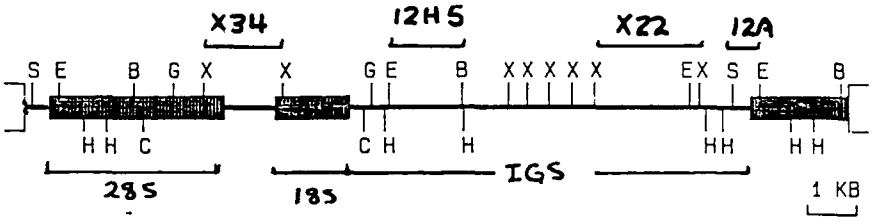
As shown below, the basis for these RFLPs are species specific differences in the EcoRI site (E) which is circled in the diagram. Also shown is lambda AGr12, the rDNA clone from which the probes were derived.



Therefore, the DNA sequence of the region containing the EcoRI site in An. gambiae should differ from the analogous region in An. arabiensis by at least one basepair. These differences would then allow the design of oligonucleotides specific for each species. Such oligonucleotide probes should then provide a clear hybridization signal for a dot blot assay. This approach has been used to advantage in other systems (21,22).

4. Experimental Results

a. Isolation of rDNA spacer sequences from An. arabiensis. In order to obtain an appropriate clone from An. arabiensis for this work, a genomic library was constructed from adult DNA. Purified DNA was partially digested with Sau3A, size separated by gel electrophoresis, and the 10-20kb fraction was eluted from the gel. The DNA was ligated with BamHI-restricted EMBL3 (Stratagene Co.), ligated with the mosquito DNA, and packaged with a Gigapack Gold kit (Stratagene Co.). A small aliquot of the library (1 x 10^6 recombinants, total) was plated and screened with intergenic spacer sequences previously obtained from An. gambiae. In order to rapidly select only those clones containing a complete rDNA repeat (18S, 28S plus spacer), a series of An. gambiae fragments, shown below, were used to probe dot blots of the plaque-purified positive clones.



These An. gambiae fragments had been subcloned in collaboration with Dr. Collins lab at the CDC. All of the fragments would be expected to hybridize in the case of a complete rDNA repeat. If a complete spacer (but not coding region) were present, EB50, X-22, and 12A would hybridize. The clones of interest to us contained X-22 which is a 2.3kb XhoI fragment which in An. gambiae contains the EcoRI site that is absent in An. arabiensis. Three clones hybridizing to all four probes were selected and amplified for further study.

These An. arabiensis recombinants were roughly mapped with a few common enzymes because it was important that we chose a typical rDNA clone for sequencing. There were two reasons for this precaution. One, from long exposures of genomic Southern blots there appeared to be some genes with unusually long spacers. These could represent differing numbers of some subrepeat within the spacer, or possibly scrambled intergenic spacers, or spacers which have diverged so much that they are actually not representative of this species. Two, some of our clones contained unusually long spacers, as judged by their length (8-13kb) and their lack of hybridization with probes 12A or X-34. Since we had carefully chosen the EcoRI site by virtue of extensive tests on field and colony material, it was important here to be certain that this region of An. gambiae would be used to design the oligonucleotide probes. One such XhoI fragment was identified and subcloned into the Bluescript vector (Stratagene Co.). In addition, the XhoI fragment (X-22) of An. gambiae was also subcloned and both were prepared for sequencing using the dideoxy chain termination method. For both species the sequence was relatively easy to obtain since it was close to one end of X-22.

b. Design of oligonucleotide probes. The sequences so obtained were analyzed using the GenePro software. The EcoRI site in An. gambiae was identified, and

the An. arabiensis sequences were then aligned. The following oligonucleotides were designed and synthesized in the Emory microchemical facility:

```
                          *
An. arabiensis 5' GAGCTCCCTAGCATTCGG 3'
                          *
   An. gambiae 5' GAGCTCCCTAGATTCGG 3'
```

The asterisk shows the (single) difference in that region. The length of the oligonucleotide was kept to a minimum in order to accentuate the one difference they bear.

c. Testing the oligonucleotide probes. Initial tests were made using a protocol which permits one to predict exactly the hybridization conditions favoring each one of the oligonucleotide probes. This protocol is described by Wood et al. (23). Oligonucleotides were end-labelled with ^{32}P and T4 kinase (21). The mosquito DNA was prepared as previously described (18). DNA from single mosquitoes was applied to nitrocellulose filters using a slot blot apparatus. Adjacent slots were filled with half of the DNA from a single mosquito. Because the test will eventually involve cutting the blot and incubating each half in a different oligonucleotide probe, half a mosquito must be shown to hybridize with these probes. The nitrocellulose filters are alkali denatured, neutralized, and air dried (25). The filters were prehybridized for 4 hrs., according to Wood's protocol, hybridized 48 hrs., at 37°C, and washed in 3M Me_4NCl, 50mM Tris-Cl, pH8, 2mM EDTA, 1mg/ml SDS at 53°C. A representative autoradiograph is shown in Figure 1..

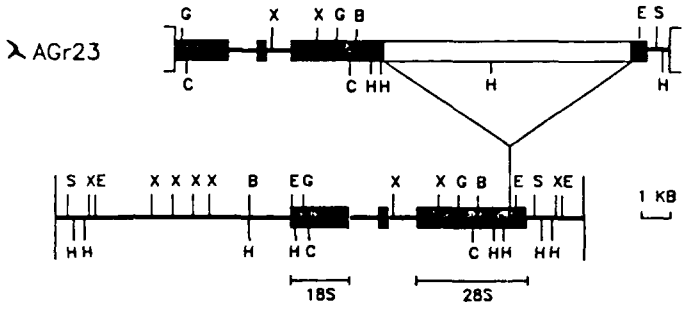
Therefore, half a single mosquito provides enough DNA for species-specific diagnosis. Dr. Collins' lab very kindly provided us with these specimens representing several geographical isolates.

The tetramethylammonium chloride (TMAC) protocol worked very well indeed and we believe this diagnostic species assay would prove to be a useful rapid method for the purpose. However, we are aware of the need to make these assays as simple as possible. Although the TMAC works perfectly, it requires a refractometer to obtain the exact concentrations required. Thus, this buffer system is quite cumbersome to prepare and it will not work unless the ion concentrations are perfectly correct. Therefore, this may be inappropriate for testing large numbers of specimens, especially under non-ideal laboratory conditions. Therefore, we experimented with various membranes (BioDyne, GeneScreen, nitrocellulose) as well as with hybridization/washing temperatures, buffer salts, and detergent concentrations. The results are of course so qualitative that they could not be adequately represented by a chart or table. However one set of conditions proved to be optimal: Mosquito DNA is prepared (18) and denatured (.3M NaOH, .6M Tris-Cl, pH 7.4, 1.5M $NaCL_2$) 5 min. The sample is divided into two aliquots and immediately applied to adjacent slots (with suction) onto prewet (20x SSC) Biodyne filter. The slots are rinsed (50μl TE with suction), dried, baked 1 hr. at 80°C. Pre-hybridization is for 4 hrs. at 37°C in 6x SSC 50mM $NaPO_4$, pH 6.8, 5x Denhardt's, 0.1mg/ml sheared calf thymus DNA, 100mg/ml dextran SO_4. Hybridization is for 48 hrs. at 37°C. The conditions for washing AGSPRI-probed filters ("A") are 2x SSC, 0.1% SDS at 51°C for 2.5 hrs. For AASPRI ("B"), are the same except for the temperature, which is 48°C. Typical results are shown in Figure 2.

From these experiments we demonstrate that the slot blot assay is a very useful and rapid means for distinguishing members of the complex as to species. The assay eliminates the need for purifying or extracting DNA; no restriction enzymes are required; and, no gel electrophoresis or blotting is necessary. Moreover, the probes appear to be specific to a single species and therefore positive identification can be made with each probe.

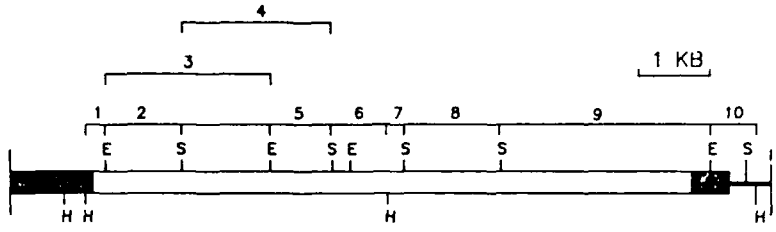
d. Possible substitute for the An. gambiae oligonucleotide probe. Since the oligonucleotide probes must be used either with TMAC protocol which is cumbersome to prepare, or at carefully controlled temperatures (differing by 3°C), we sought to enhance or replace the An. gambiae probe with a longer species-specific sequence. This sequence derives from an intervening sequence (IVS) found in some An. gambiae rDNA genes.

During earlier studies on the An. gambiae clones, one (lambda AGr23) appeared to have an IVS in its 28S coding region. It is shown below with lambda AGr12 for comparison:



Such IVS have been found in several other Diptera (17) and they are potentially highly variable compared to the coding regions. Thus we wished to determine whether this IVS was present in many or few of the gambiae rDNA repeats, to see if it was common enough to give a strong hybridization signal. Given this we wished to determine whether portions of the IVS could be absent in other species in the gambiae complex. Thus, a Southern blot containing DNA of various members of the complex was probed with fragment 12A which includes spacer and 28S coding region. Such a blot is shown in Figure 3. The expected 1.3kb HindIII fragment is present in all species, but other longer fragments are also revealed. These longer fragments represent rDNA genes which bear IVSs in the 28S coding region. Interestingly other Dipterans show IVS in this same general vicinity of the 28S.

In order to determine whether our An. gambiae IVS-containing clone (lambda AGr23) contained species-specific fragments, fragments of the IVS were subcloned by Dr. Collins' lab. The fragments are illustrated below:



Each fragment was used to (separately) probe genomic Southerns, and the results are shown in Table 2. There are several An. gambiae IVS fragments which do not hybridize to An. arabiensis, An. merus, or An. quadriannulatus, although they do hybridize with An. melas. Nonetheless, these fragments are excellent probes for dot blots and can be used alone or to augment the detection of An. gambiae, s.s.

5. Conclusions

The oligonucleotide probes developed for use in a rapid dot blot assay to identify An. gambiae and An. arabiensis are found to work very well and should adequately serve the purpose. In addition, An. gambiae-specific probes were also found and tested. These could be useful to determine the proportion of gambiae in a mixed population and/or to augment the use of the oligonucleotides.

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Table 1. Designation, geographic origin, and source of mosquito colonies used in the present study. Sources: 1, London School of Hygiene and Tropical Medicine; 2, Istituto di Parassitologia, Universita di Roma; 3, Kenya Medical Research Institute; 4, the South African Institute for Medical Research; 5, colonized at the Centers for Disease Control (CDC), Atlanta, Georgia.

Species and Designation	Geographic Origin	Source
An. gambiae, G-3	The Gambia	1
An. gambiae, GMMK6	Burkino Faso	2
An. gambiae, As46	Kenya	5
An. gambiae, Zan	Zanzibar	1
An. arabiensis, Arzag	Burkino Faso	2
An. arabiensis, Senn	Sudan	1
An. arabiensis, Kisu	Kenya	3
An. arabiensis, Man	South Africa	4
An. arabiensis, Gmal	Sudan	4
An. melas, Bal	The Gambia	5
An. merus, V-12	Kenya	5
An. merus, Zulu	Zululand	4
An. quadriannulatus, Chil.	Zimbabwe	5

Table 2. Southern hybridization pattern of IVS fragments from clone lambda-AGr23 with the indicated mosquito strains. The location of the probe fragments is shown in the text. The + indicates the presence of one or more IVS fragments; the * indicates the presence of the expected 0.8kb HindIII fragment; and blanks indicate no test was performed. Probe 11 is fragment 12A, shown in Figure 1.

Mosquito Strain	**Probe Number**										
	1	2	3	4	5	6	7	8	9	10	11
An. gambiae, G-3	* +	+	+	+	+	+	+	+	* +	* +	* +
An. gambiae, GMMK6	* +	+	+	+	+	+	+	+	* +	* +	* +
An. gambiae, As46	* +	+	+	+	+	+	+	+	* +	* +	* +
An. gambiae, Zan	* +	+	+	+	+	+	+	+	* +	* +	* +
An. arabiensis, Arzag	* +	-	-	-	-		-	+	* +	* +	* +
An. arabiensis, Senn	* +	-	-	-	-	-	-	+	* +	* +	* +
An. arabiensis, Gmal	* +						-	+			* +
An. arabiensis, Kisu		-	-	-	-	-		+	* +	* +	
An. arabiensis, Man		-	-	-	-	-	-	+	* +	* +	
An. melas, Bal	* +	-	+	+	+	+	-	+	* +	*	* +
An. merus, Zulu	* +										* +
An. merus, V-12	* +	-	-	-	-	-	-	+	* +	*	*
An. quadriannulatus, Chil	* +	-	-	-	-	-	-	+	* +	*	* +

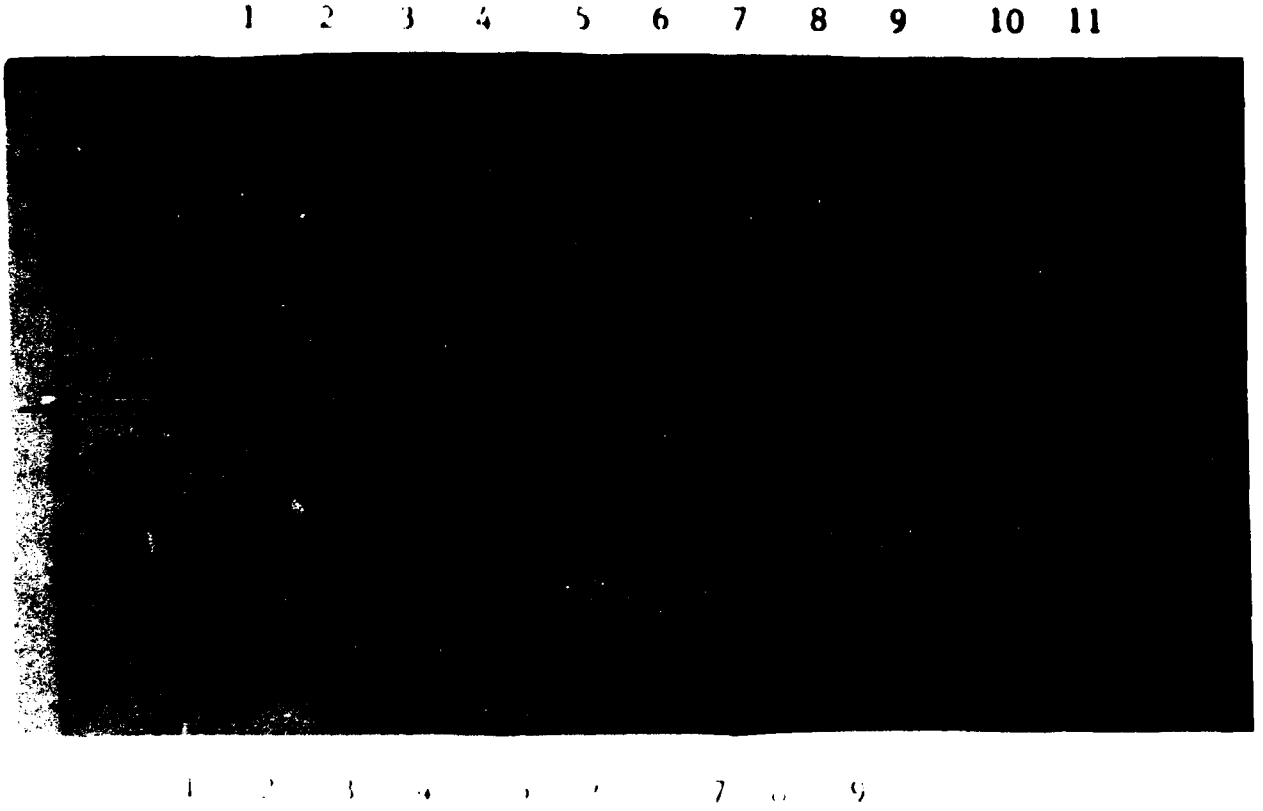

Figure 1. Slot blots hybridized with species-specific oligonucleotide probes.

Differential hybridization is based upon altering the concentration of tetramethylammonium chloride. Two slot blot strips are shown: the upper strip hybridized with the An. gambiae-specific oligonucleotide and the lower strip hybridized with the An. arabiensis-specific oligonucleotide. For these blots, adjacent slots were filled with half of a single mosquito homogenate. The origin of the strains is given in Table 1. Upper strip: Wells 1,2: G3 females; wells 3,4: G3 males; well 5: GMMK6 male; well 6: Arzag female; well 7: Arzag male; well 8: V-12 female; well 9: V-12 male; well 10: Zan female; well 11: Zan male. Lower strip: Well 1: G3 female; well 2, G3 male; well 3: GMMK6 male; well 4: Arzag female; well 5: Arzag male; well 6, V-12 female; well 7: V-12 male; well 8, Zan female; well 9, Zan male.

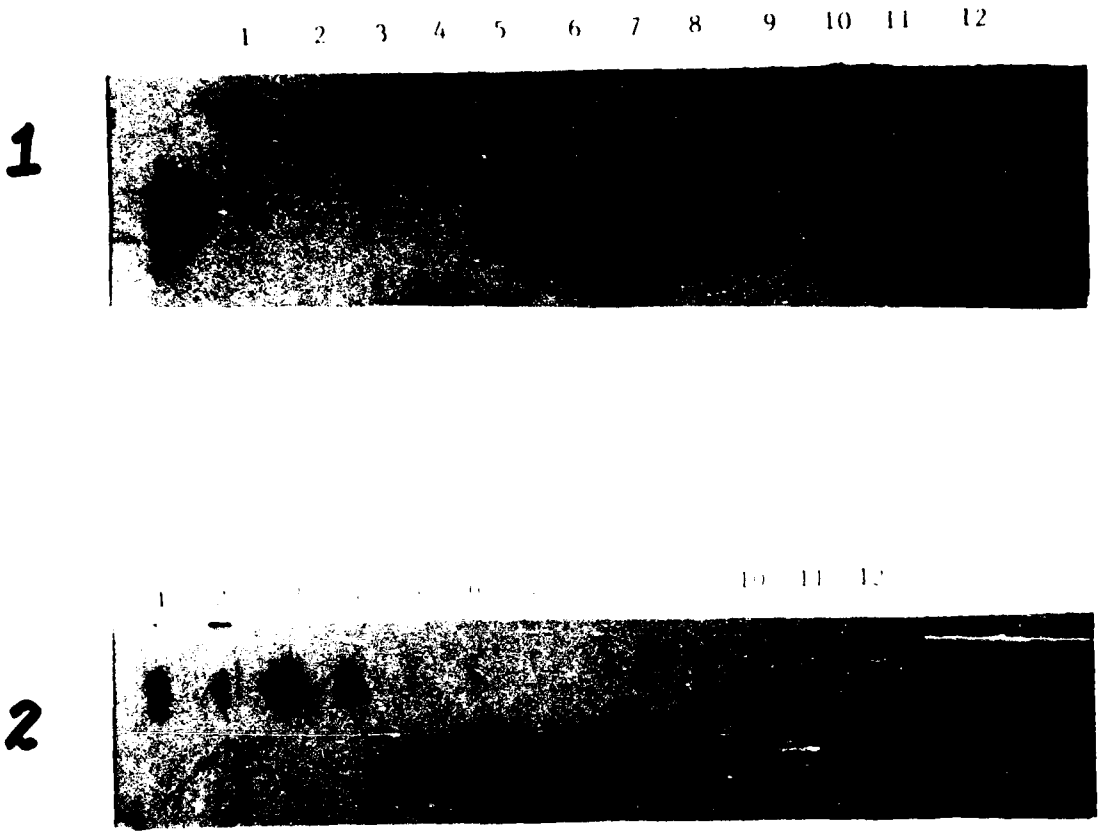

Figure 2. Slot blots hybridized with species-specific oligonucleotide probes.

Differential hybridization is based upon altering wash temperature. Two experiments are displayed. For each experiment the upper half of the strip was hybridized with the An. gambiae-specific probe and the lower half hybridized with the An. arabiensis-specific probe. Well 1, A.g. G-3; Well 2, A.g. GMMK6; Well 3, A.g. AS46; Well 4, Zan; Well 5, A.a. Arzag; Well 6, A.a. Senn; Well 7, A.a. Man; Well 8, GMAL; Well 9, A. melas Bal; Well 10, A. merus Zulu; Well 11, A. merus V12; Well 12, A. quad. Chil. The source of these geographical isolates is given in Table 1.

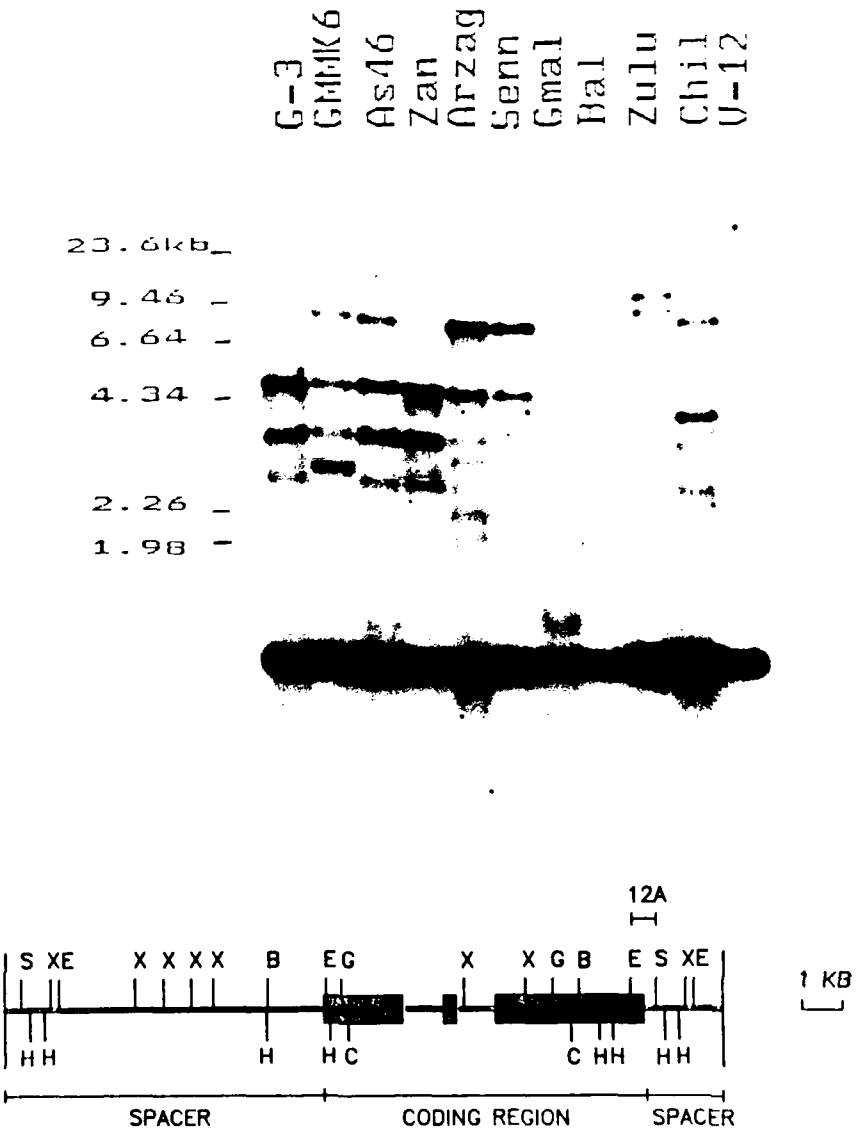

Figure 3. Southern analysis of An. gambiae complex mosquitoes.

lanes 1-4, An. gambiae; lanes 5-7, An. arabiensis; lane 8, An. melas; lanes 9-10, An. merus; lane 11, An. quadriannulatus. The origin of mosquito strains is given in Table 1. The restriction map in the lower part of the figure is lambda AGr12 (18). The probe was the EcoRI/SalI fragment shown at th;e 3' end of the 28S coding region. E, EcoRI; S, SalI; H, HindIII; X, XhoI; B, BamHI; G, BglII; C, SstII.

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3. Finnerty V, SM Paskewitz, MO Rasmussen & FH Collins. A dot blot assay for distinguishing members of the An. gambiae complex based upon oligonucleotide probes. J Med Vet Entomol, submitted.

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